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Low Affinity Binding Site Clusters Confer Hox Specificity and Regulatory Robustness

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SUMMARY

In animals, Hox transcription factors define regional identity in distinct anatomical domains. How Hox genes encode this specificity is a paradox, because different Hox proteins bind with high affinity in vitro to similar DNA sequences. Here, we demonstrate that the Hox protein Ultrabithorax (Ubx) in complex with its cofactor Extradenticle (Exd) bound specifically to clusters of very low affinity sites in enhancers of the *shavenbaby* gene of *Drosophila*. These low affinity sites conferred specificity for Ubx binding in vivo, but multiple clustered sites were required for robust expression when embryos developed in variable environments. Although most individual Ubx binding sites are not evolutionarily conserved, the overall enhancer architecture—clusters of low affinity binding sites—is maintained and required for enhancer function. Natural selection therefore works at the level of the enhancer, requiring a particular density of low affinity Ubx sites to confer both specific and robust expression.

INTRODUCTION

Diversity along the anterior-posterior axis of animals results from differential expression of Hox transcription factors, which regulate different sets of target genes to determine the features specific to each anatomical region (McGinnis and Krumlauf, 1992). For example, in *Drosophila*, *Sex combs reduced* (*Scr*) determines anterior thoracic segments (Struhl, 1982; Wakimoto and Kaufman, 1981), whereas *Ultrabithorax* (*Ubx*) and *abdominalA* (*abdA*) specify thoracic and abdominal segments (Lewis, 1978; Sánchez-Herrero et al., 1985).

Hox protein specificity is paradoxical, because all Hox proteins have similar DNA binding domains (the homeodomain), particularly for residues that contact DNA directly (Akam, 1989; McGinnis and Krumlauf, 1992). As a result, all Hox proteins bind similar DNA sequences with high affinity (Berger et al., 2008; Mann et al., 2009; Noyes et al., 2008). In principle, one solution to this paradox is that sequences outside of the homeodomain, which have diverged among Hox proteins, allow interactions with a diversity of cofactors to confer specificity. However, only two cofactors, the homeodomain proteins Extradenticle/Pbx (Exd) and Homothorax/MEIS (Hth) (Moens and Selleri, 2006), are known to interact with Hox proteins (Chan et al., 1994; Chang et al., 1995; Mann et al., 2009). Exd dimerizes with Hox proteins and Hth facilitates nuclear localization and DNA binding of Exd (Pai et al., 1998; Rieckhof et al., 1997; Ryoo et al., 1999). Thus, Hox specificity is unlikely to arise from interactions with a diversity of cofactors.

However, Hox protein structure is altered when bound to DNA with Exd, resulting in increased binding site specificity of Hox-Hth-Exd complexes in comparison with Hox monomers (Joshi et al., 2007; Slattery et al., 2011). In vivo support for this latent specificity model came from studies of artificial enhancers containing multimerized Hox-Exd binding sites (Ryoo and Mann, 1999). Therefore, it is not clear whether this mechanism is sufficient to account for the high degree of regulatory specificity exhibited by Hox proteins on native enhancers.

One clue that may inform the Hox specificity paradox is that many enhancers, including Hox-regulated enhancers, contain multiple binding sites for the same transcription factor (Arnone and Davidson, 1997; Gotea et al., 2010; Lifanov et al., 2003; Ochoa-Espinosa et al., 2005; Papatsenko et al., 2002; Stanojevic et al., 1991). These so-called homotypic binding site clusters are widespread, but the functions of clustered binding sites are understood in only a few cases. For example, homotypic clusters can fine-tune the response to graded transcription factors levels (Driever et al., 1989; Gaudet and Mango, 2002; Jiang and Levine, 1993; Rowan et al., 2010; Struhl et al., 1989), control the timing of enhancer activation (Gaudet and Mango, 2002), or determine whether binding results in repression or activation (Ramos and Barolo, 2013). However, elimination of individual binding sites

in homotypic clusters often has little or no effect on enhancer activity (Doniger et al., 2005; Driever and Nüsslein-Volhard, 1989; Hersh and Carroll, 2005; Saramäki et al., 2006; Stanojevic et al., 1991), suggesting that there may be additional reasons for the widespread existence of homotypic binding site clusters.

To gain insight into the Hox specificity paradox, we asked how Hox factors regulate native enhancers to achieve a specific pattern of epidermal trichomes along the anterior-posterior axis of *Drosophila* larvae. Trichome patterns display strong differences between adjacent segments in a Hox-dependent manner (Lewis, 1978; Sánchez-Herrero et al., 1985). Because *shavenbaby* (*svb*) is the master control gene for trichome development (Chanut-Delalande et al., 2006; Delon et al., 2003; Payre et al., 1999), we examined whether and how Hox factors regulate *svb*. We found that *svb* enhancers are directly regulated by Ubx and that they solve the Hox specificity paradox by employing clusters of low affinity Ubx-Exd binding sites. Specificity is encoded by low affinity sites and homotypic clusters of these sites provide regulatory robustness. This overall architecture—homotypic clusters of low affinity binding sites—is evolutionarily conserved and may provide a general mechanism to reconcile the need for both enhancer specificity and robustness.

RESULTS

Ubx Positively Regulates *svb* Expression

In wild-type embryos of *Drosophila melanogaster*, cells of the ventral first abdominal segment (A1) differentiate a row of stout trichomes (Figure 1B). These trichomes were lost in the absence of *Ubx* (Figure 1D). Reciprocally, ectopic expression of *Ubx* using a heat shock inducible promoter (*HS:Ubx*) caused production of ectopic trichomes in thoracic segments (Figure 1F) (González-Reyes and Morata, 1990; Mann and Hogness, 1990). Because *svb* controls trichome development (Chanut-Delalande et al., 2006), we tested whether *Ubx* regulates *svb* expression. In wild-type embryos, *svb* was expressed strongly in cells of A1 and other abdominal segments that generate ventral trichomes and only weakly in the third thoracic segment (Figure 1A). In the absence of *Ubx*, *svb* expression was reduced in segment A1 (Figure 1C), consistent with the loss of the A1 trichomes in these larvae (Figure 1D). When we expressed *Ubx* ubiquitously, *svb* was upregulated in thoracic segments in a pattern similar to *svb* expression in segment A1 (Figure 1E). These results indicate that *Ubx* is required for expression of *svb* in the cells that generate A1 trichomes and that *Ubx* is sufficient to induce ectopic expression of *svb* when misexpressed in thoracic segments.

Ubx Controls Multiple *svb* Enhancers

To determine how *Ubx* regulates *svb* expression, we examined the effects of altered *Ubx* expression on two *svb* enhancers, called *E* and *7*, that drive ventral stripes of expression (Figure 1G) (Frankel et al., 2010, 2011; McGregor et al., 2007). Through systematic functional dissection, we identified a 292 bp region of *E*, called *E3N*, and a 1,056 bp region of *7*, called *7H* (Figure S1 available online; Table S1), that each drove expression that accurately recapitulated the ventral patterns generated by the larger regions from which they were derived.

In wild-type embryos, *E3N* and *7H* reporter genes were expressed in ventral rows of segments A1–A8 (Figures 1H and 1I). In embryos that lacked *Ubx*, *E3N* and *7H* reporter gene expression was lost in the A1 segment (Figures 1J and 1K) and reduced in A2–A8 segments (Figures 1H–1K), consistent with the reduction in trichome numbers caused by loss of *Ubx* function (Lewis, 1978). Ectopic *Ubx* caused ectopic expression of *E3N* and *7H* in thoracic segments and increased expression in abdominal segments (Figures 1L and 1M). In response to all manipulations of *Ubx* function, the expression patterns driven by *E3N* and *7H* were similar to endogenous *svb* expression (Figures 1A–1F and 1H–1M). Therefore, these two enhancers respond to Ubx and, at least in part, capture the regulatory inputs of Hox genes to establish the anterior-posterior pattern of *svb* expression and trichomes.

Hox proteins bind DNA with Exd and Hth (Mann et al., 2009) and embryos lacking either *hth* or *exd* display homeotic transformations of trichome patterns (Jürgens et al., 1984; Peifer and Wieschaus, 1990; Rieckhof et al., 1997). To test if the Exd-Hth complex contributes to Ubx regulation of *svb* expression, we assayed expression of the *E3N* and *7H* enhancers in embryos homozygous for a strongly hypomorphic *hth* allele, hth^{P2}, which cannot facilitate nuclear localization of Exd (Noro et al., 2006; Rieckhof et al., 1997). *E3N* and *7H* expression was abrogated in hth^{P2} embryos (Figures 1N and 1O), suggesting that Ubx requires Exd and Hth for activation of these *svb* enhancers.

The loss of *E3N* and *7H* activity in abdominal segments in hth^{P2} embryos suggests that multiple Hox genes activate these enhancers. While *Ubx* specifies the trichomes in A1, it acts together with *abdA* to specify trichomes in more posterior segments (Lewis, 1978). Ubx and AbdA have similar DNA-binding specificities in complex with Exd (Karch et al., 1990; Slattery et al., 2011) and either *Ubx* or *abdA* is sufficient to drive *svb* expression in ventral abdominal stripes (Coiffier et al., 2008). Accordingly, we found that embryos deficient for *Ubx* and *abdA* expressed neither *E3N* nor *7H* in abdominal stripes (see below), indicating that both Ubx and AbdA activate the *E3N* and *7H* *svb* enhancers.

Ubx Regulates the *E3N* and *7H* Enhancers Directly through Multiple Low Affinity Binding Sites

To determine whether Ubx binds to *svb* enhancers, first we examined genome-wide Ubx and Hth chromatin immunoprecipitation data (Choo et al., 2011). These data revealed in vivo binding of Ubx and Hth at the *E3N* and *7H* regions, as well as at other *svb* enhancer regions (Figure S1). These results suggest that Ubx may regulate the *E3N* and *7H* *svb* enhancers directly, which we tested further below.

Surprisingly, the DNA sequences of *E3N* and *7H* contained no Hox-Exd sites that match those previously identified by systematic evolution of ligands by exponential enrichment sequencing (SELEX-seq) (Slattery et al., 2011). Therefore, we systematically searched for Ubx binding sites in *E3N* using electrophoretic mobility shift assays (EMSAs) of overlapping DNA fragments (Figures 2A and S2; Table S2). Ubx showed concentration-dependent binding to *E3N1* and *E3N2* fragments, but only when in complex with both Hth and Exd (Figures 2C, S2B, and S2C). These fragments bound Ubx-Exd in complex with either full-length Hth (Hth^{FL}) or with Hth^{HM}, similar to a naturally

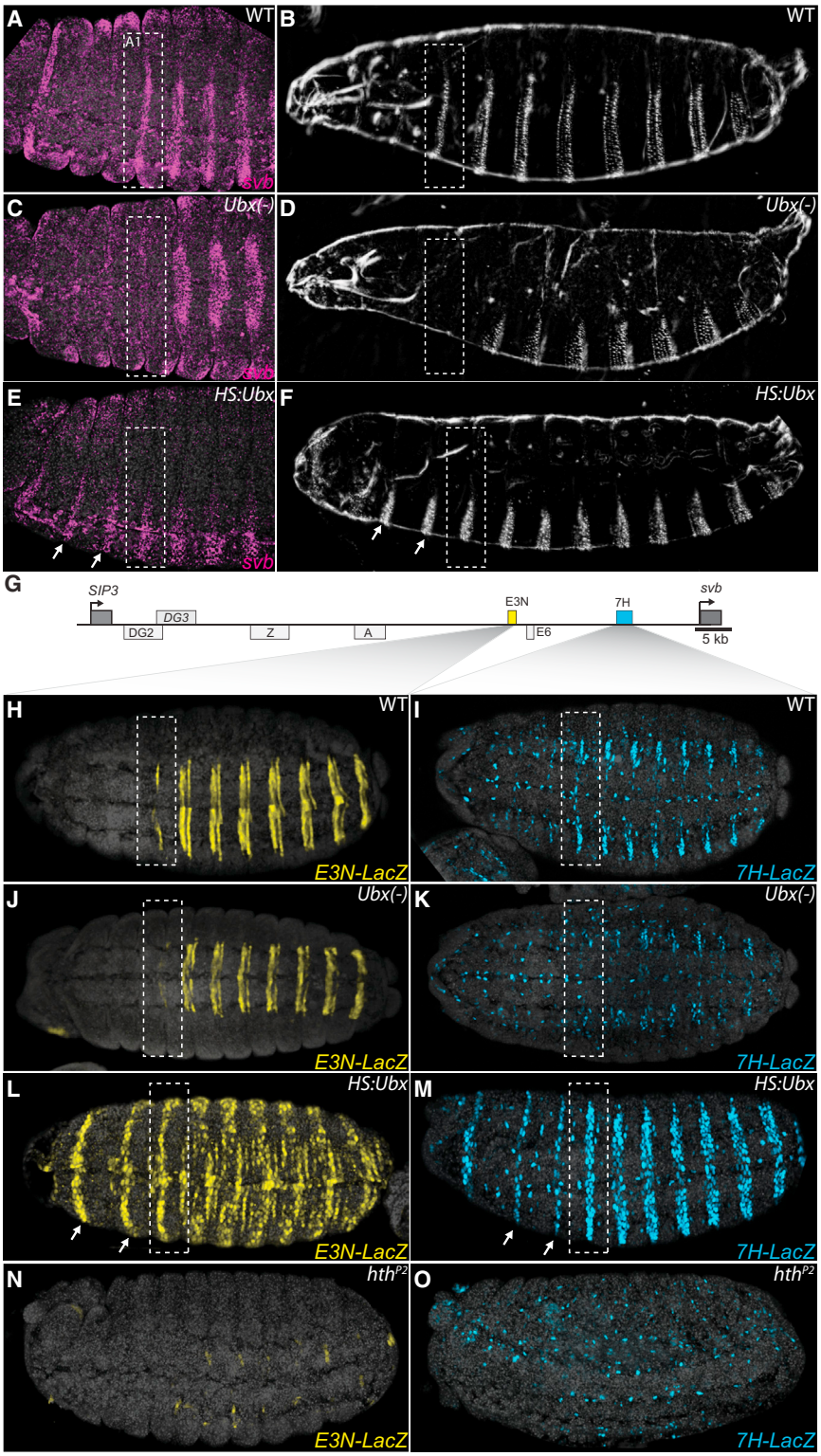


Figure 1. Ubx Is Necessary and Sufficient for *svb* Expression

(A–F) Embryos stained with fluorescent *svb* mRNA probe and larval cuticle preps (B, D, and F) of the indicated genotypes. Loss of *Ubx* function transformed segment A1 into a thoracic segment that lacks *svb* expression (C) and larval trichomes (D), highlighted with bounding boxes. Ubiquitous expression of Ubx protein resulted in homeotic transformations of thoracic segments (arrows) into segments resembling segment A1 (E and F).

(G) Schematic of the *svb* upstream *cis*-regulatory region, indicating embryonic enhancers. The ventral enhancers *E3N* and *7H* are highlighted in yellow and blue boxes, respectively. See also Figure S1.

(H–O) Expression of *E3N*::*lacZ* or *7H*::*lacZ* reporter constructs (I, K, M, and O). *Ubx* was necessary for *E3N* and *7H* reporter expression in segment A1 (J and K) and sufficient for their expression in thoracic segments when expressed ubiquitously (L and M).

(N and O) In hth^{P2} mutant embryos, activity of both the *E3N* and *7H* enhancers was lost.

See also Table S1.

occurring isoform of Hth that lacks a homeodomain but that can translocate Exd to the nucleus (Ryoo et al., 1999). Neither Hth^{HM}-Exd nor Hth^{FL}-Exd bound to these *E3N* subfragments in the absence of Ubx (Figures S2B and S2C). Thus, despite the absence of predicted Ubx binding sites in *E3*, these data revealed binding of Ubx-Exd-Hth trimers—hereafter abbreviated as Ubx-Exd for simplicity—to several regions of this enhancer.

To identify the Ubx-Exd binding sites in the *E3N* subregions, we systematically tested binding of Ubx-Exd to oligonucleotides mutated at each 5′-AT dinucleotide pair (Figure S3) and found that most of the Ubx-Exd binding activity came from three sites (Figure 2C). Mutation of each of two binding sites in *E3N1* reduced Ubx-Exd binding and mutation of both together abolished Ubx-Exd binding (Figure 2C, see also Figure S3). In the *E3N2* fragment, we found a third site that, when mutated, abolished Ubx-Exd binding (Figures S3P and S3Q). Mutation of an additional site located near the 5′ end of *E3N1* also reduced Ubx-Exd binding, suggesting that this region may contain another low affinity Ubx-Exd binding site (Figures S2 and S3D). The Ubx-Exd binding sites in *E3N* show variable levels of evolutionary conservation and only site 3 is conserved across all sequenced *Drosophila* species.

We next tested, in vivo, the role of Ubx-Exd sites identified in vitro by generating transgenic constructs with all possible combinations of the three sites mutated (Figure 2). Mutation of

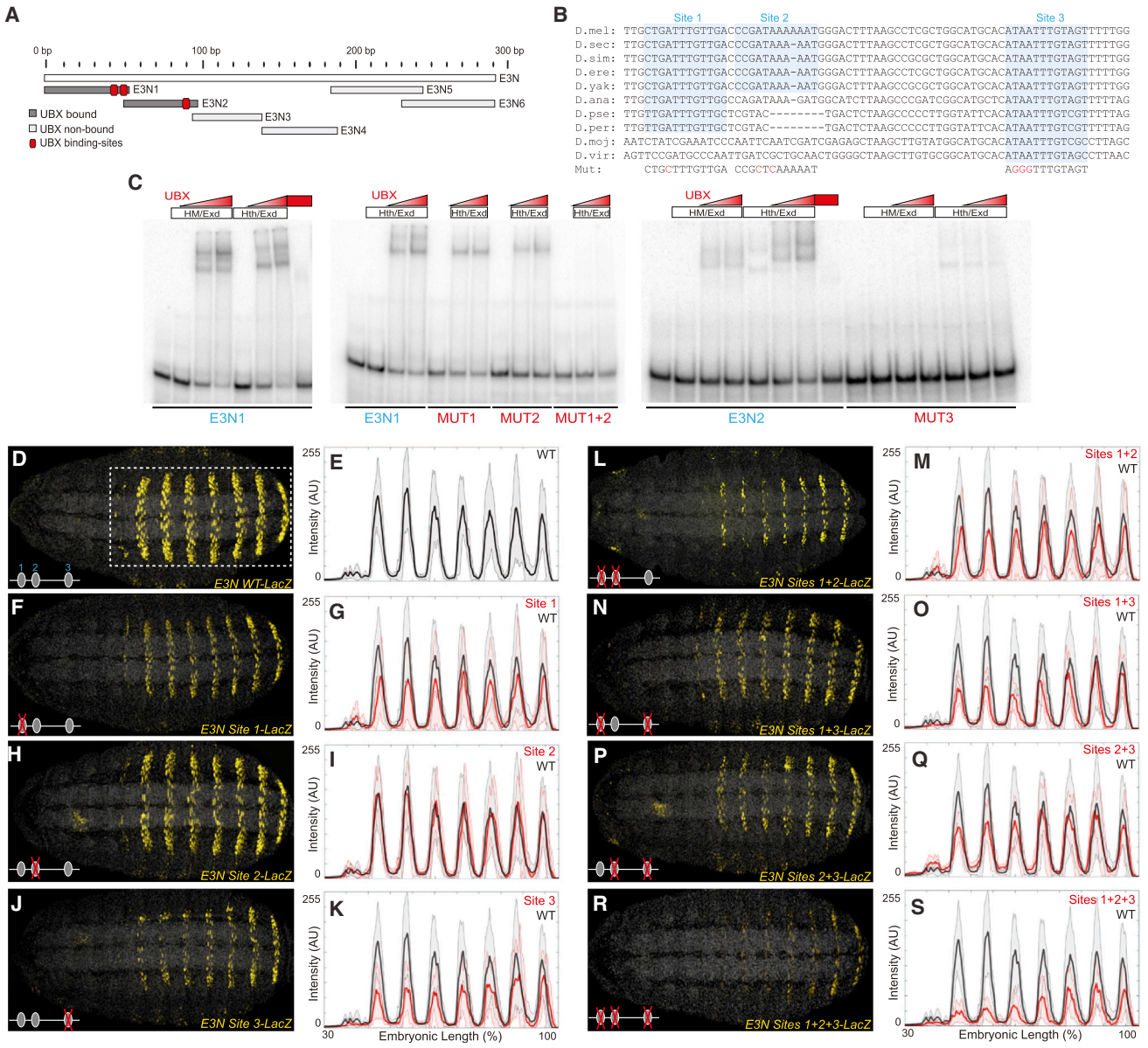


Figure 2. The *svb E3N* Enhancer Contains a Cluster of Ubx-Exd Binding Sites

(A) A schematic of the regions tested for their ability to bind Ubx-Exd, assayed via EMSAs. See also Figures S2 and S3.

(B) Sequence alignment for the region of the *E3N* enhancer containing the three Ubx-Exd sites, labeled and highlighted. Dashes indicate gaps in the aligned sequence. Mutations of the Ubx-Exd binding sites are shown (Mut).

(C) Ubx-Hth-Exd bound specifically to each of the three sites, as demonstrated with EMSAs. In this and the following figures, Hth and HM refer to the full-length (Hth^{FL}) and homeodomainless (Hth^{HM}) isoforms of Hth, respectively.

(D–S) Expression of *E3N*::*lacZ* reporter constructs with Ubx-Exd sites altered as indicated (B), juxtaposed with plots of average expression in the region outlined in (D) (n = 10 for each genotype). In all plots, the black and red lines denote expression driven by the wild-type and modified enhancers, respectively. Shaded areas indicate ±1 SD. AU, arbitrary units of fluorescence intensity.

See also Figures S4 and S5 and Tables S1 and S2.

either site 1 or site 3 reduced the expression levels driven by *E3N* (Figures 2F, 2G, 2J, and 2K). Mutation of site 2 had no detectable effect on *E3N* expression (Figures 2H and 2I), including when combined with either site 1 or site 3 mutations (Figures 2L, 2M, 2P, and 2Q). However, when both site 1 and site 3 were mutated, the *E3N* enhancer still drove weak expression, which was reduced further upon knockout of site 2 (Figures 2N, 2O, 2R, and 2S). Thus, all three Ubx-Exd sites in the *E3N* enhancer are functional in vivo.

We obtained very similar results for Ubx binding to the *7H* enhancer. In vitro assays identified three low affinity Ubx-Exd binding sites in *7H* (Figures S4A and S4B). Individual mutation

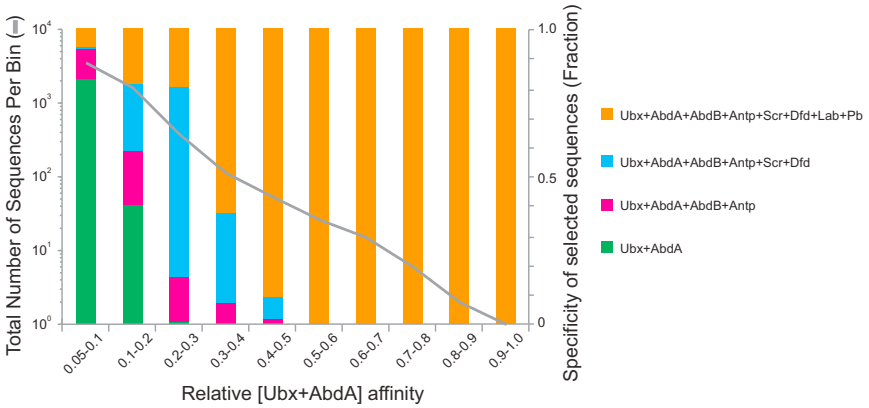


Figure 3. Inverse Correlation between Sequence Affinity and Specificity

The proportion of 12mer sequences bound by various Hox-Exd complexes versus relative affinity of these 12mers for Ubx/AbdA-Exd is shown as colored bars (specificity groups). The number of 12mers in each affinity bin is plotted as a gray line. Average relative affinities of 12mers were calculated for four pairs of Hox-Exd complexes with similar binding profiles: (1) Labial and Pb, (2) Dfd and Scr, (3) AbdB and Antp, and (4) Ubx and AbdA. Sequences specific for Ubx/AbdA-Exd (green bars) are more prevalent in lower affinity bins than in higher affinity bins.

of each of the three Ubx-Exd binding sites did not modify the activity of *7H* in embryos, either qualitatively (Figures S4C, S4G, S4K, and S4O) or quantitatively (Figures S4D, S4H, S4L, and S4P). In contrast, simultaneous mutation of sites 1 and 2, or sites 2 and 3, decreased *7H* activity (Figures S4E–S4N) and mutation of all three sites almost completely abrogated *7H* expression (Figures S4Q and S4R). Collectively, these results indicate that, as observed for *E3N*, the *7H svb* enhancer uses at least three low affinity Ubx-Exd sites to drive expression along the AP axis of embryos. The absence of Ubx-Exd sites in *E3N* or *7H* that match those detected by SELEX-seq (Slattery et al., 2011) implies that these sites have very low affinity for Ubx-Exd (see below).

In addition to *Ubx*, the *E3N* and *7H* enhancers are regulated in vivo by *abdA* (Figure S5). Therefore, we tested whether the Ubx-Exd sites we identified could also bind AbdA-Hth-Exd (AbdA-Exd). In vitro, AbdA-Exd bound to the same *E3N* and *7H* binding sites as Ubx-Exd did, and binding was abrogated when these sites were mutated (Figure S5). Thus, Ubx-Exd and AbdA-Exd directly regulate the *E3N* and *7H* enhancers through the same binding sites.

Taken together, these data show that both the *E3N* and *7H svb* enhancers contain clusters of low affinity Ubx/AbdA-Exd binding sites that are required to drive *svb* expression in ventral abdominal stripes. They further indicate that these sites mediate the action of Ubx in segment A1 and Ubx plus AbdA in segments A2–A8.

Proper Regulation of a *svb* Enhancer Requires Low Affinity Ubx Binding Sites

While our in vivo assays demonstrated that all of the Hox-Exd sites in *E3N* and *7H* are required for proper function, it is not clear why these enhancers employ low affinity rather than higher affinity binding sites. We hypothesized that the low affinity of these binding sites may be part of the solution to the Hox specificity paradox. To explore this idea, we analyzed previously published data in which the DNA sequence preferences of all *Drosophila* Hox-Exd complexes were measured using SELEX-seq, resulting in relative affinity scores from 0.03 to 1 (Slattery et al., 2011). Using these data, we asked if there was any correlation between affinity and specificity. For example, do sequences with low affinity versus high affinity for Ubx-Exd display preference for Ubx-Exd compared to other Hox-Exd complexes? The results of this analysis were striking; only sequences with a relative affinity lower than 0.3 bound Ubx/AbdA-Exd specifically compared to the other Hox-Exd complexes (Figure 3). Moreover, as the relative affinity for Ubx/AbdA-Exd decreased, the number of sequences that bound specifically to Ubx/AbdA-Exd increased (Figure 3). These data imply that Hox-Exd complexes display, at least in vitro, a tradeoff between binding affinity and specificity.

To test whether this affinity-specificity tradeoff holds in vivo, we generated *E3N* transgenic variants in which we varied the affinity of the Ubx-Exd binding sites according to the relative affinities predicted by SELEX-seq (Figure 4A) (Slattery et al., 2011). Although none of the binding sites found in the native *svb* enhancer were identified by SELEX-seq, we estimate (based on the core 8-mer) that they have relative affinities <0.03 (Slattery et al., 2011). Every mutation that increased the affinity of Ubx-Exd sites resulted in qualitative or quantitative changes in *E3N* enhancer expression (Figure 4). For example, converting either native sites 1 or 2 to high-affinity sites (scores of 0.87 or 0.79, respectively) resulted in increased expression in the normal domain of *E3N* and ectopic expression anteriorly and dorsally (Figures 4C and 4D). Replacing site 3 in *E3N* with the highest affinity site (score of 1.0) also resulted in ectopic expression in anterior segments and in the intestine (Figure 4B). We further explored the functional consequences of gradually increasing the affinity of a Hox-Exd binding site by replacing site 3 with sites that have a range of relative affinities, from 0.06 to 0.72. A small increase in affinity to 0.06 resulted in higher levels of *E3N* expression within its normal expression domain (Figures 4H and 4I). Increasing the affinity to 0.25, 0.65, and 0.72 altered levels of expression in the normal domains of *E3N* and induced ectopic expression in anterior segments (white arrows in Figures 4E–4G).

We also observed strong position effects of a high affinity site, similar to observations in a previous study (Swanson et al., 2010). Placing the highest affinity site 5′ of the *E3N* enhancer resulted in ectopic expression in anterior segments, but decreased expression in the normal domain (Figure S6B). In contrast, placing this site inside the enhancer increased expression in the normal domain and generated ectopic expression in multiple regions (Figure S6C). We cannot rule out the possibility, however, that

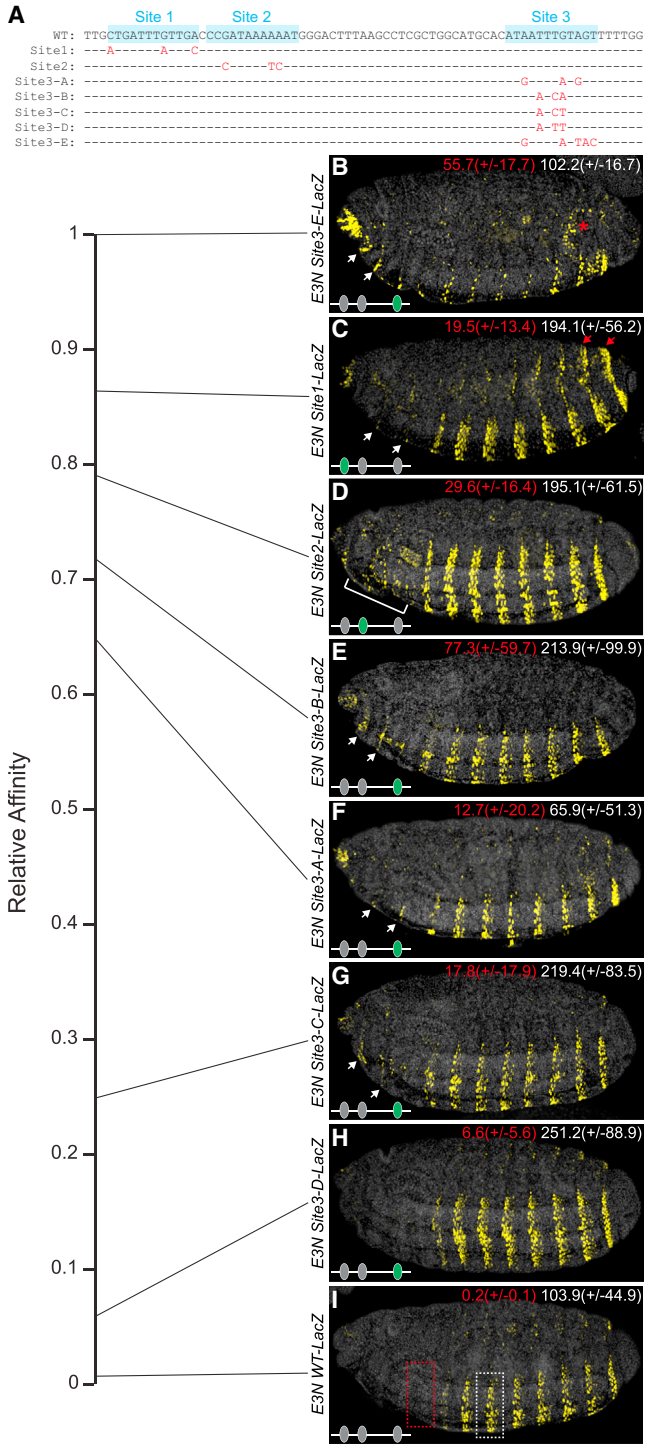


Figure 4. Conversion of Low Affinity Ubx-Exd Binding Sites to Higher Affinity Sites Results in Ectopic Expression

(A) Aligned *E3N* sequences from wild-type and mutated sequences. Dashes and red letters indicate unaltered and modified sequence, respectively.

(B–I) Embryos carrying *E3N::lacZ* constructs, with Ubx-Exd sites altered as indicated in (A). The numbers in the top right of each panel indicate the average levels of expression in the regions outlined in (I) (n = 10 for each genotype), measured in arbitrary units of fluorescence intensity. Numbers in parantheses indicate ±1 SD. White arrows and brackets denote expression in domains anterior to segment A1 (B–G). The red asterisk marks ectopic staining in the intestine; red arrows indicate ectopic dorsal and lateral expression (C). See also Figure S6 and Table S1.

these position-dependent effects resulted from the creation or destruction of binding sites for additional factors.

Taken together, these results indicate that Hox-Exd sites with higher affinity than the native sites alter the specificity of the *svb* enhancer, demonstrating that the affinity-specificity tradeoff that was inferred from in vitro data also pertains in vivo.

High Affinity Hox Binding Sites Decreased the Specificity of Enhancer Function

Replacement of native sites with high affinity sites caused ectopic expression mostly outside of the domains of *Ubx* and *abdA* expression (Figure S5), suggesting that these high affinity sites bound transcription factors other than Ubx and AbdA. Indeed, in embryos deficient for *Ubx*, the *E3N* enhancers with high affinity binding sites showed the expected reduction of expression in A1 (where *Ubx* is the only Hox gene active), but they also continued to drive ectopic expression in anterior segments (Figures 5A–5D).

Sex comb reduced (*Scr*) was an attractive candidate for driving some of the ectopic anterior expression of *svb* enhancers carrying high-affinity sites. Scr is expressed in anterior segments (Kuroiwa et al., 1985) and SELEX-seq data indicated that Scr-Exd can bind to high-affinity Ubx/Exd binding sites (Slattery et al., 2011). When assayed on the *E3N svb* enhancer, Scr-Exd showed little or no in vitro binding to the native *E3N* sites, but it bound to the high affinity sites even more strongly than Ubx-Exd bound to the native sites (Figure 5E). In vivo, uniform expression of *Scr* produced no obvious changes in the expression of wild-type *E3N* (Figures 5F and 5G), but drove ectopic expression of *E3N* variants that carried one high-affinity site (Figures 5H–5K). Thus, replacing low affinity Ubx/AbdA-Exd sites with high-affinity sites enabled the *E3N* enhancer to respond to Scr. In addition to Scr, it is likely that other homeodomain transcription factors bind and activate the *E3N* enhancers carrying high-affinity sites to generate their broad domains of ectopic expression.

Together, our results indicate that the native low affinity Ubx/AbdA-Exd binding sites in the *E3N* enhancer confer specificity for Ubx-Exd and AbdA-Exd over other Hox proteins, such as Scr, and probably over additional homeodomain factors.

Clusters of HOX Binding Sites Confer Robustness to Genetic and Environmental Variability

As discussed earlier, some of the Ubx/AbdA-Exd binding sites in the *E3N* and *7H* enhancers can be mutated with minimal effects on reporter gene expression (Figures 2 and S4). It is not clear, therefore, why these enhancers contain multiple Hox binding sites. We wondered if the multiple, apparently redundant, Ubx-Exd binding sites within individual *svb* enhancers contribute to transcriptional robustness, in the same way that multiple enhancers of *svb* confer robustness in the face of environmental and genetic variation (Frankel et al., 2010).

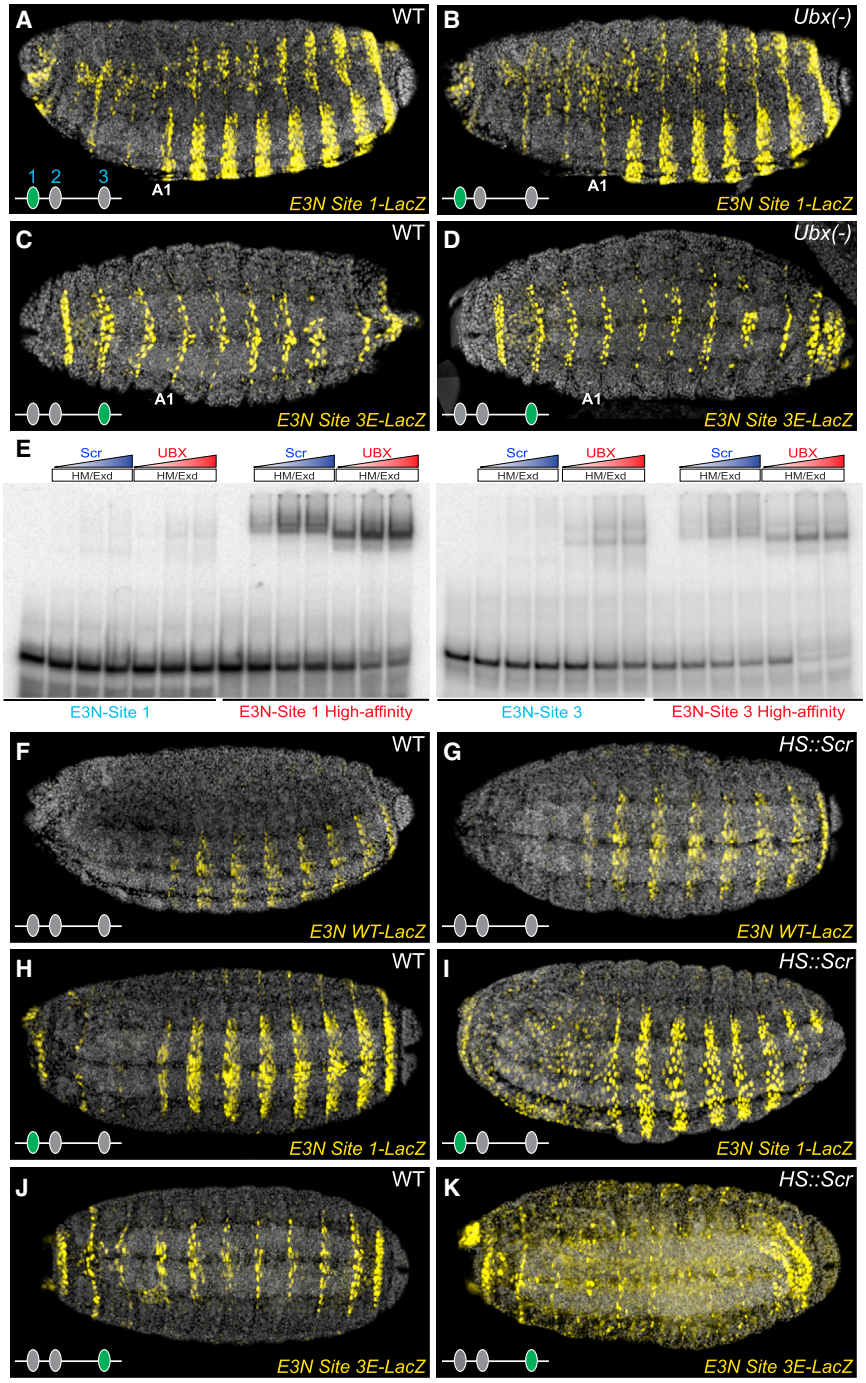


Figure 5. Low Affinity Ubx-Exd Binding Sites Provide High Ubx-Exd Specificity

(A–D) Embryos carrying *E3N*::*lacZ* constructs, with Ubx-Exd sites altered as indicated in (Figure 4A). In embryos deficient for Ubx, *E3N*::*lacZ* with high affinity sites drove extensive ectopic expression (B and D).

(E) Scr-Exd did not bind to wild-type *E3N* Ubx-Exd sites in vitro, as demonstrated with EMSAs. However, both Scr-Exd and Ubx-Exd bound to high-affinity Ubx-Exd sites.

(F–K) Ubiquitous expression of Scr (*hs*::*Scr*) did not alter expression of the wild-type *E3N*::*lacZ* (G), but caused ectopic expression of *E3N*::*lacZ* carrying high-affinity Ubx-Exd sites (I and K).

See also Tables S1 and S2.

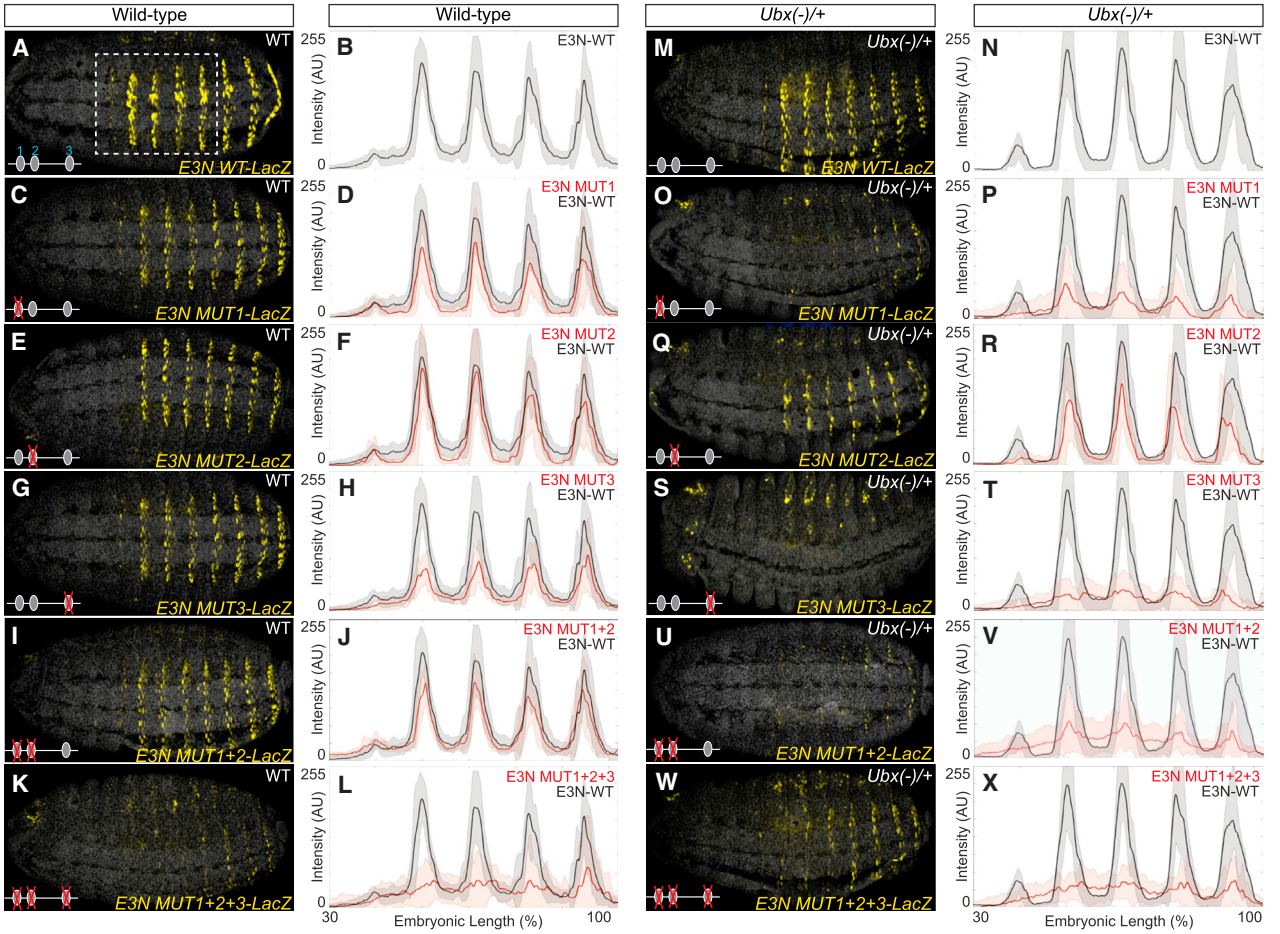
To test this hypothesis, first we examined the effects of altered levels of Ubx on the expression of *E3N* enhancers. The wild-type *E3N* enhancer drove normal expression in embryos heterozygous for an *Ubx* null mutation (Figures 6A, 6B, 6M, and 6N). In contrast, all *E3N* enhancers that contained single mutations in the Ubx-Exd binding sites drove dramatically lower levels of expression in *Ubx* heterozygotes, compared to wild-type embryos (Figures 6C–6T). Similar effects were observed for most combinations of the mutations (Figures 6I, 6J, 6U, and 6V). These results indicate that *E3N* requires multiple sites to confer robustness when *Ubx* dose is perturbed. The reduced activity of these *E3N* enhancers in *Ubx* heterozygotes also provides further evidence that the Ubx-Exd binding sites respond to Ubx in vivo.

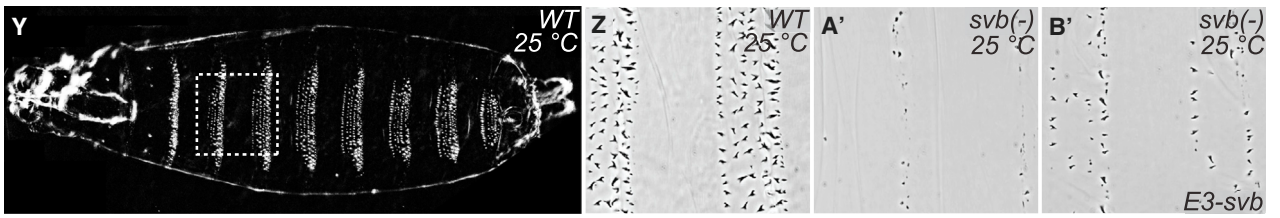
Next, we assayed the effects of environmental variation on enhancer activity by exploiting the fact that *svb* enhancers driving a *svb* cDNA in *svb* null embryos provide a sensitive and quantitative readout of enhancer function (Frankel et al., 2010, 2011). We reared embryos at 17°C and 32°C, temperature extremes that are still compatible with normal development (Powsner, 1935). In embryos carrying the wild-type *E3N*::*svb* rescue construct, trichome numbers were relatively invariant to temperature extremes (Figure 6C′). In contrast, enhancers containing a single mutated Ubx/Exd binding site showed reduced rescue of trichomes at extreme temperatures (Figure 6C′). Furthermore, the simultaneous mutation of site 1 and 2 abrogated trichome rescue at extreme temperatures, while other double or triple combinations led to no rescue (Figure 6C′).

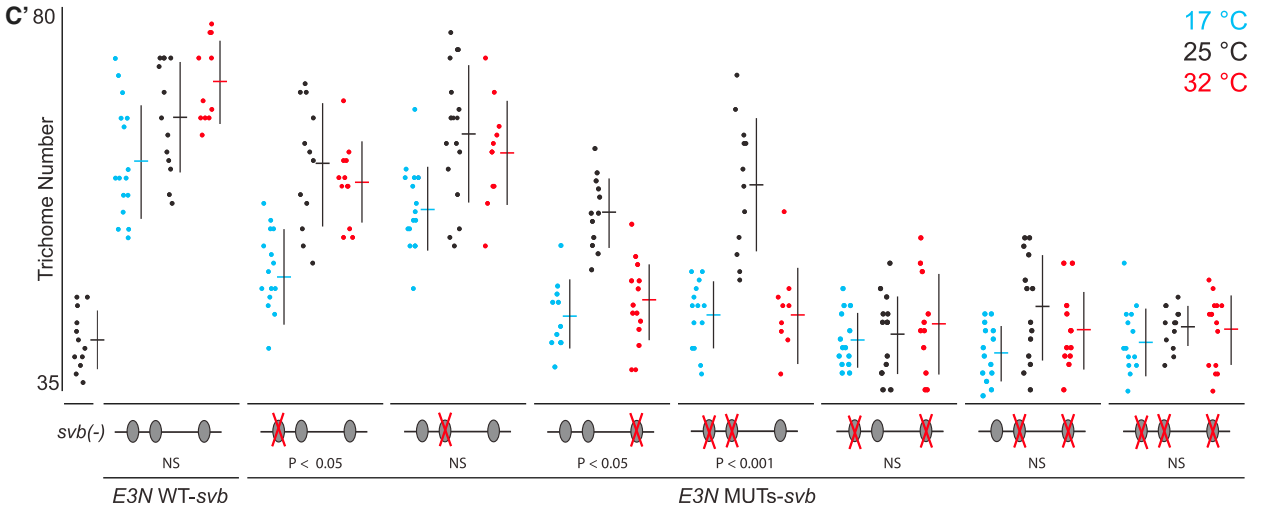
These results indicate that multiple Ubx-Exd binding sites are required for normal enhancer function and to cope with variable genetic backgrounds and environments, similar to conditions faced by flies in the wild.

Ubx Binds a Cluster of Low Affinity Binding Sites in the Orthologous *E3N* Enhancer from a Distantly Related Species

We wondered whether the enhancer architecture discovered for *E3N* and *7H*, with homotypic clusters of low affinity Hox-Exd







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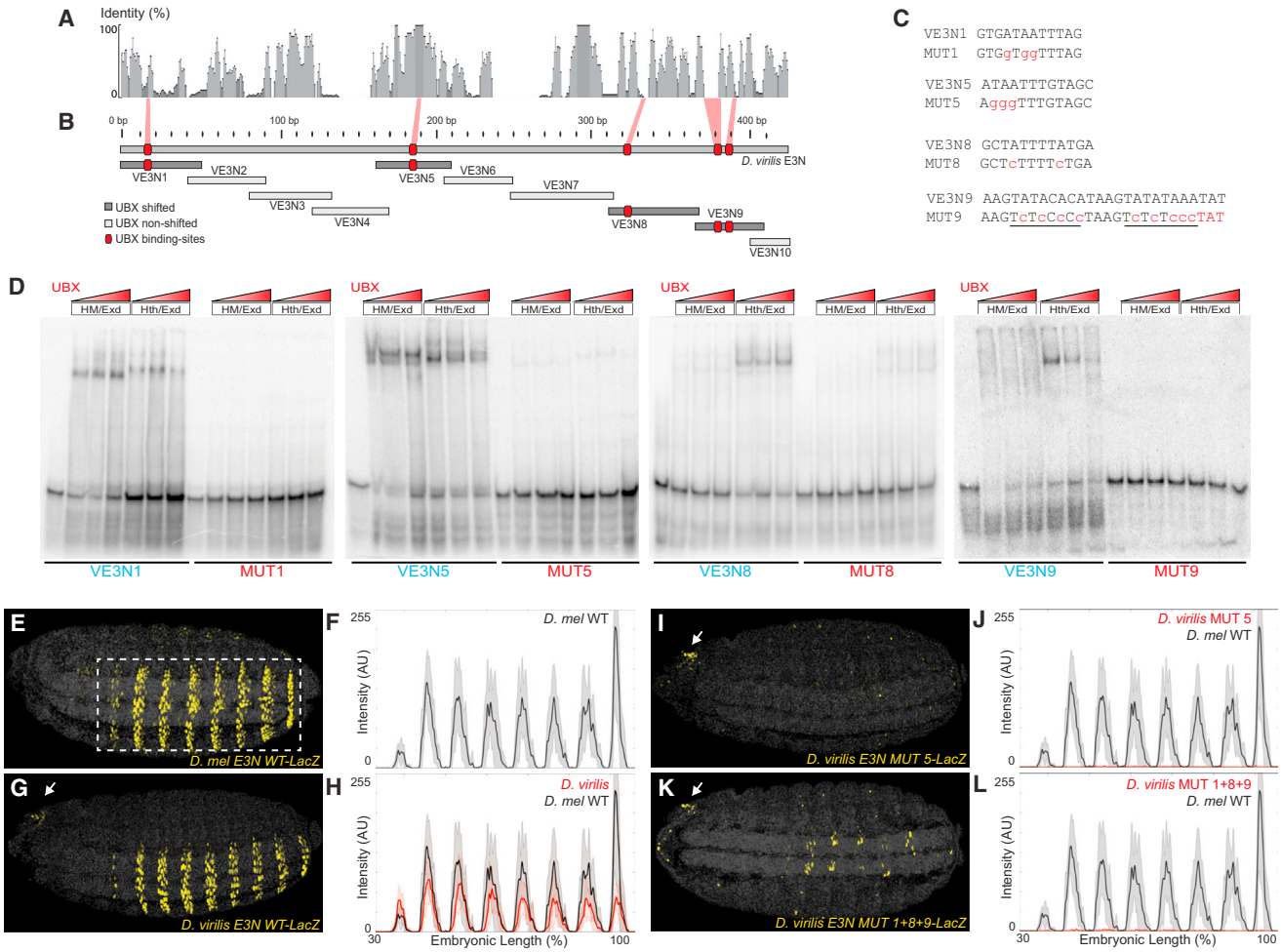

Figure 7. Multiple Low Affinity Poorly Conserved Ubx-Exd Binding Sites Regulate the *Drosophila virilis E3N* Enhancer

(A) Sequence conservation over a 10 bp sliding window for a sequence alignment of the *E3N* region from ten *Drosophila* species.

(B) Regions tested for the ability to bind Ubx-Exd, assayed via EMSAs (see also Figure S7). The positions of the Ubx-Exd sites are indicated with red boxes.

(C) *E3N* Ubx-Exd binding-site sequences aligned with site-specific mutations indicated in lowercase, red letters.

(D) Ubx-Hth^{FL}-Exd and Ubx-Hth^{HM}-Exd bound five sites in the *D. virilis E3N* enhancer, as demonstrated with EMSAs (see also Figure S7). This binding was reduced when the sites were mutated (MUT).

(E–L) Embryos carrying *E3N*::*LacZ* constructs, with Ubx-Exd sites altered as indicated in (C), juxtaposed with plots of average expression (n = 10 for each genotype). Black lines denote expression driven by the *D. melanogaster* and *D. virilis* enhancers, respectively.

See also Tables S1 and S2.

sites, is an evolutionarily conserved feature of *svb* enhancers. Because the large-scale *cis*-regulatory landscape of *svb* has been well conserved in *Drosophila virilis* (Frankel et al., 2012), we examined this question by focusing on the *E3N* region of *D. virilis*.

Drosophila melanogaster and *D. virilis* last shared a common ancestor ~40 mya and the *E3N* region displays little sequence conservation between these species (Figure 7A). We thus employed EMSAs to identify, in an unbiased manner, all of the Ubx-Exd binding sites putatively present in the *D. virilis E3N*

Figure 6. The *svb E3N* Enhancer Contains a Cluster of Ubx-Exd Binding Sites that Confer Robustness against Environmental and Genetic Variation

(A–X) Wild-type (A–L) and *Ubx* heterozygote (M–X) embryos carrying *E3N*::*lacZ* constructs with Ubx-Exd sites altered as indicated in Figure 2B, juxtaposed with plots of average expression in the region outlined in (D) (n = 10 for each genotype). Shaded bounding areas indicate ±1 SD. AU, arbitrary units of fluorescence intensity.

(Y–B′) Cuticle preps showing that the *E3N*::*svb* transgene (B′) in a *svb* null mutant background rescued a subset of the wild-type trichome pattern (cf. Y–A′).

(C′) The number of trichomes in the larval A2 segment for the corresponding genotypes. The error bars indicate ±1 SD. Significance values are sequential Bonferroni test p values, to control the type I error rate, from separate ANOVA tests for each genotype.

See also Table S1.

orthologous region (*VE3N*). We found that four fragments—*VE3N1*, *VE3N2*, *VE3N5*, and *VE3N9*—bound Ubx-Exd in vitro (Figures 7B–7D). Comprehensive mutagenesis of these fragments revealed five Ubx-Exd binding sites (Figures 7B, 7C, and S7). One of these sites is evolutionarily conserved and four sites display no sequence conservation to *D. melanogaster* and only weak conservation to closely related species (Figure 7A). As observed for *D. melanogaster*, none of the Ubx-Exd binding sites of *VE3N* were detected by SELEX-seq, indicating that they are low affinity sites.

We next tested whether these Ubx-Exd sites function in vivo by generating transgenic *D. melanogaster* lines that contained either the wild-type or mutated *D. virilis VE3N* enhancers. The wild-type *VE3N* enhancer drove lower levels of expression than did its *D. melanogaster E3N* counterpart (Figures 7E–7H), a result observed often in tests of orthologous enhancers (Crocker et al., 2008; Ludwig et al., 1998). Mutation of the conserved site present in *VE3N* resulted in the loss of reporter expression (Figures 7I and 7J). To test if only this site was required for V*E3N* expression, we mutated the four nonconserved sites, leaving the conserved site intact. This reporter also displayed very little *VE3N* activity (Figures 7K and 7L). Therefore, multiple Ubx-Exd binding sites, at least some of which are poorly conserved, contribute to the proper regulation of the *D. virilis VE3N* enhancer.

Taken together, these results indicate that clustering of low affinity Ubx-Exd sites is an evolutionarily conserved strategy used by *svb* enhancers, although many of the individual binding sites are not conserved across species.

DISCUSSION

We have demonstrated that the Hox protein Ubx regulates separate enhancers of the *svb* gene by binding, with its cofactors Exd and Hth, to clusters of low affinity binding sites. Combining in vitro and in vivo assays, we provided experimental demonstration of an affinity-specificity tradeoff for Hox proteins, such that enhancers that integrate Hox inputs to drive regionalized expression are unlikely to utilize high affinity Hox binding sites. Forced to utilize low affinity sites, enhancers have evolved to contain multiple binding sites to ensure regulatory robustness to genetic and environmental variations. Most individual Ubx-Exd sites have evolved rapidly, but evolution has conserved overall enhancer architecture, with clusters of low affinity sites.

Homotypic clusters of transcription factor binding sites are pervasive in animal genomes (Arnone and Davidson, 1997; Gotea et al., 2010; Lifanov et al., 2003; Ochoa-Espinosa et al., 2005; Papatsenko et al., 2002; Stanojevic et al., 1991) and several models have been proposed to explain their existence (Doniger et al., 2005; Giorgetti et al., 2010; He et al., 2012; Segal et al., 2008). Our results provide experimental evidence that homotypic clusters of Hox binding sites can confer robustness to enhancers. This may reflect a more widespread phenomenon. Although many enhancers contain homotypic clusters with low affinity sites, previous studies have rarely detected changes in expression by deleting individual binding sites (Doniger and Fay, 2007; Driever and Nüsslein-Volhard, 1989; Estella et al., 2008; Giorgianni and Mann, 2011; Hersh and Carroll, 2005; Saramäki et al., 2006; Stanojevic et al., 1991). However, these mutated enhancers have not been tested in variable environments. It is possible that many of these clustered sites confer regulatory robustness.

It is useful to compare our results with previous studies that have demonstrated specific regulatory functions for homotypic clusters. For example, clustered binding sites in an enhancer of the *Drosophila hunchback* gene mediate cooperative DNA binding by Bicoid, which provides threshold-dependent enhancer activity (Driever et al., 1989; Lebrecht et al., 2005; Struhl et al., 1989). In other cases, clusters of homotypic binding sites act in a noncooperative manner to allow enhancers to respond in a graded fashion (Giorgetti et al., 2010), for example to determine expression levels in response to transcription factor concentrations (Driever et al., 1989; Gaudet and Mango, 2002; Rowan et al., 2010). It is worth noting that in these cases, where homotypic clusters mediate specific linear or nonlinear outputs, enhancers are bound by transcription factors that belong to small paralogous families: e.g., two paralogs for Msn2 (Hasan et al., 2002); three for p53 (Belyi et al., 2010); two for Dorsal (Silverman and Maniatis, 2001); and five for NFkB (Silverman and Maniatis, 2001). In contrast, there are 84 homeodomain-containing proteins encoded in the *Drosophila* genome, many with overlapping specificities (Berger et al., 2008; Noyes et al., 2008). Therefore, in previously described examples of homotypic clusters, binding affinity may not be a strong constraint on specificity.

For the Hox regulated *svb* enhancers, low affinity Ubx/AbdA-Exd binding sites enable specificity, while the clustering of low affinity sites confers phenotypic robustness. This is a fundamentally different constraint on clustered binding sites than observed in all previous examples. The affinity-specificity tradeoff, initially supported by our computational analysis of in vitro data, was confirmed in vivo by progressively increasing the affinity of the Ubx-Exd binding sites. While replacement of low affinity sites with higher affinity sites always quantitatively altered enhancer activity, either positively or negatively, most higher affinity sites generated strong ectopic expression. As we show, this ectopic expression is driven, at least in part, by gaining the binding of additional Hox proteins, which are normally not involved in the regulation of these enhancers. Other studies have performed replacement of low affinity sites with higher affinity sites and, in some cases, they have also observed ectopic expression (Busser et al., 2012; Driever et al., 1989; Gaudet and Mango, 2002; Jiang and Levine, 1993; Peterson et al., 2012; Ramos and Barolo, 2013; Scardigli et al., 2003; Stewart-Ornstein et al., 2013; Struhl et al., 1989). These altered patterns of expression may reflect increased sensitivity of enhancers to the same transcription factor that binds to the wild-type enhancer (Jiang and Levine, 1993). We observed a similar effect for Ubx and AbdA-dependent upregulation of *svb* enhancers in the cells in which they are normally expressed. In addition, however, we found that sites with higher affinity resulted in a reduced specificity, due to the binding of additional homeodomain proteins, such as Scr, to *svb* enhancers. Our computational analyses suggest that this affinity-specificity tradeoff is a fundamental property of Hox proteins and would therefore influence the architecture of enhancers that must generate specific outputs in response to Hox factors. We suggest that transcription factors

that belong to other large paralog groups may exhibit a similar affinity-specificity tradeoff and that enhancers regulated by these factors may also exploit clusters of low affinity sites.

Our results help to explain previous difficulties with bioinformatic prediction of functional Hox binding sites, because low affinity sites are difficult to detect reliably. Indeed, the low affinity sites that implement Hox regulation within *svb* enhancers share little similarity with canonical Hox or Hox-Exd binding sites. Consequently, a very large number of seemingly disparate DNA sequences can confer low affinity binding for Hox proteins. If Hox-Exd sites are often clustered in the genome, then signals from genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) will reflect binding to the entire cluster (as we observed) and the signals associated with individual low affinity sites may be difficult to discern from noise. Identification of important low affinity sites will require a change in computational approaches to analyzing genome-wide data. Currently, it is de rigueur to apply an arbitrary threshold to genome-wide data and then to analyze only signals above this threshold. This approach is likely to bias detection toward high affinity sites, whose functions may be distinct from those of clusters of low affinity sites.

Our findings provide insight into how different Hox proteins regulate specific target genes to generate phenotypic diversity across the anterior-posterior axis. One unanswered question is how the many low affinity DNA sequences, which appear to share little in common, are bound by the same Hox-Exd complex with apparently similar affinity. It is possible that variations in DNA shape (deviations from the structure of canonical B-DNA) influence Hox-Exd binding to low affinity sites (Dror et al., 2014; Joshi et al., 2007; Rohs et al., 2009). It remains unclear if very different sequences can adopt similar shapes, or whether instead the Hox-Exd complex can recognize a range of shapes. Resolution of this question will require structural studies of Hox-Exd complexes bound to a range of low affinity DNA sequences and quantitative analysis of their binding dynamics in vivo.

EXPERIMENTAL PROCEDURES

Fly Strains and Transgenic Constructs

DNA fragments were cloned into the reporter constructs *placZattB* and *pHSPattB GFP* and the *pRSQsvb* rescue construct (Frankel et al., 2011) (see Table S1). Mutations were introduced using site-directed mutagenesis (Genescript). Plasmids were integrated into the *attP2* landing site by Rainbow Transgenic Flies. Additional strains used were: svb^{R9}/*FM7c twi::GFP* (Delon et al., 2003); *HS::Ubx-1;* Ubx^{1}; hth^{P2} (Noro et al., 2006); and $Ubx^{1}abdA^{D24}AbdB^{D18}$ (Bloomington stock 1108).

Embryo Staining and Cuticles

Stage 15/16 embryos were collected, fixed, and stained using standard protocols with mouse anti-βGal (1:1,000, Promega) and anti-mouse AlexaFluor (1:500, Invitrogen) antibodies. Cuticles were prepared following standard protocols, imaged with phase-contrast microscopy, and ventral trichomes in larval A2 segments were counted.

Image Analysis

Embryos carrying reporter constructs were imaged on a Leica SPE Confocal Microscope. Sum projections of confocal stacks were assembled, images were scaled, background was subtracted using a 50-pixel rolling-ball radius and plot profiles of fluorescence intensity were analyzed using ImageJ software (http://rsb.info.nih.gov/ij). Data from the plot profiles were analyzed further in MATLAB (http://www.mathworks.com) (Crocker and Stern, 2013).

In Vitro Affinity-Specificity Tradeoff Calculations

Average relative affinities of 12mers from SELEX-seq data (Slattery et al., 2011) were calculated for four pairs of Hox-Exd complexes that share similar binding preferences: (1) Labial and Pb, (2) Dfd and Scr, (3) Antp and AbdB, and (4) Ubx and AbdA. Specificity groups (colored bars in Figure 3) were defined as having an average relative affinity ≥ 0.05 for bound complexes and <0.03 for unbound complexes. The proportion and total number of sequences in each specificity group were calculated for ten bins based on their Ubx/AbdA-Exd relative affinities.

DNA Alignments

Multiple sequence alignments were performed using Geneious (http://www.geneious.com) with MUSCLE alignment algorithms (anchor optimized).

Protein Purification and EMSAs

Ubx (isoform IVa), *abdA*, Hth^{HM}*-Exd*, and Hth^{FL}*-Exd* constructs, protein purification, and EMSA conditions were described previously (Lelli et al., 2011). Further experimental details are provided in Table S2.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.11.041.

AUTHOR CONTRIBUTIONS

The experimental plan was conceived by J.C., N.A., R.S.M., and D.L.S. Most in vivo assays were performed by J.C. All in vitro assays and computational analysis were performed by N.A., assisted by L.R. and R.S.M. A.P.M., A.A., P.V., S.P., F.P., and D.L.S. defined the minimal enhancer elements. N.F. and S.W. performed some in vivo assays. J.C. assembled all figures. J.C., R.S.M., and D.L.S. wrote the paper, with input from N.A., A.P.M., N.F., S.W., S.P., and, especially, from F.P.

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